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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 39/00, 35/14, C12P 21/06
G01N 33/558, C07K 15/00, 13/00
C07H 15/12

A1

(11) International Publicati n Number: WO 92/13559

(43) International Publication Date: 20 August 1992 (20.08.92)

(21) International Application Number: PCT/US92/01152

(22) International Filing Date: 10 February 1992 (10.02.92)

(30) Priority data:
654,205 8 February 1991 (08.02.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 654,205 (CIP)
Filed on 8 February 1991 (08.02.91)

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CD4-GAMMA1 AND CD4-IgG1 CHIMERAS

(57) Abstract

This invention provides an expression vector encoding a CD4-gamma1 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer.

histocompatibility complex (MHC) class II molecules on the surface f antigen-presenting cells to mediat fficient cellular immune response interactions. In man, CD4 is also the target of interaction with the human immunodeficiency virus (HIV).

HIV infects primarily helper T lymphocytes and monocytes/macrophages, cells that express surface CD4, leading to a gradual loss of immune function which results in the development of the human acquired immune deficiency syndrome (AIDS). The initial phase of the HIV replicative cycle involves the high affinity interaction between the HIV exterior envelope glycoprotein gp120 and surface CD4 (Kd approximately 4 x 10^{-9} M) (2). Several lines of evidenc demonstrate the requirement of this interaction for viral infectivity. In vitro, the introduction of a functional cDNA encoding CD4 into human cells which do not express CD4 is sufficient to render otherwise resistant cells susceptible to HIV infection (3). In vivo, viral infection appears to be restricted to cells expressing CD4. Following the binding of HIV gp120 to cell surface CD4, viral and target cell membranes fuse, resulting in the introduction of the viral capsid into the target cell cytoplasm.

Characterization of the interaction between HIV gp120 and CD4 has been facilitated by the isolation of cDNA clones encoding both molecules (4, 5). CD4 is a nonpolymorphic, lineage-restricted cell surface glycoprotein that is a member of the immunoglobulin gene superfamily. High-level expression of both full-length CD4 and truncated, soluble versions of CD4 (sCD4) have been described in stable expression systems. The availability of large quantities of purified sCD4 has permitted a detailed understanding of the structure of this complex glycoprotein. Mature CD4 has a relativ molecular mass (Mr) of 55 kilodaltons and consists

blocks the intercellular spread of virus from HIV-infected cells to uninfected cells by inhibiting virus-mediated cell fusion (1, 13).

In addition to in vitro results, experiments with sCD4 in simian immunodeficiency virus (SIV)-infected rhesus monkeys have been described. These studies demonstrated that administration of 2 milligrams (intramuscular) of sCD4 for 28 days to SIV-infected rhesus monkeys led to a decreased ability to isolate virus from peripheral blood lymphocytes and bone marrow. In addition, the growth of granulocyte-macrophage and erythrocyte progenitor colonies in the bone marrow returned to normal levels. These data suggest that administration of sCD4 to SIV-infected rhesus monkeys leads to a diminution of the viral reservoir.

Phase I human clinical trials demonstrated that there is no significant toxicity or immunogenicity associated with administration of sCD4 at doses as high as 30 mg/day. Pharmacokinetic studies revealed the serum half-life of sCD4 to be 45 minutes following intravenous administration, 9.4 hours after intramuscular dosing, and 10.3 hours after the drug was given subcutaneously (14, 15). Preliminary antiviral studies were inconclusive with respect to CD4 cell count and levels of HIV antigen. Because the maximum tolerated dose was not reached, the antiviral effect of sCD4 may have been underestimated, especially in light of recent data concerning differences in sCD4 concentrations required to inhibit laboratory strains of HIV-1 compared to primary viral isolates (16).

Although these in vitro, primate, and human clinical studies with sCD4 have produced encouraging results, they have also defined several limitations. First, the measured serum half-life of sCD4 is relatively short. Second, sCD4 is monovalent with respect to gp120 binding in contrast with

d velopments combining the t chniques of molecular genetics with monoclonal antibody technology has lead to th production of "humanized" chimeric antibodies in vitro. In these chimeric antibodies, the variable domains of human immunoglobulin heavy and light chains are replaced with specific heavy and light chain variable domains from a murine monoclonal antibody (17-19). The result of this genetic manipulation is a molecule with specificity for a particular antigen and the characteristics of human immunoglobulins.

Sequence and structural analyses of CD4 indicate that the four extracellular domains are immunoglobulin-like. Since the Fc portion of immunoglobulins controls the rate of catabolism of the molecules (serum half-life ranging from 14 to 21 days) and provides various effector functions, several reports describe the replacement of variable and constant domains of immunoglobulins with the immunoglobulin-lik domains of CD4 (21-24).

CD4-IgG1 heavy chain fusion proteins resulting in chimeric gamma1 heavy chain dimers have been described (21). These molecules contain the gamma1 heavy chain CH1 domain in addition to the hinge, CH2 and CH3 domains. However, heavy chain assembly and secretion from mammalian cells is less efficient if the CH1 domain is expressed in the absence of light chains (25). Subsequently, a CD4-IgG1 heavy chain fusion protein lacking the CH1 domain and the first five amino acids of the hinge region was described which was secreted to high levels (22). These fusion proteins retain various effector functions of immunoglobulin molecules, such as Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC) toward HIV-1-infected cells, and placental transfer via an Fc receptor-dependent mechanism (22). CD4-IgM heavy chain fusion proteins have also been described (26). In addition, CD4-IgG1 fusion proteins have

been described wherein th V1V2 domains f CD4 are fused to the CH1, hinge, CH2 and CH3 d mains of a gamma1 heavy chain, and wherein the V1V2 domains of CD4 are fused to th constant domain of a kappa light chain (29).

Fusion proteins linking CD4 to toxins have also been constructed and tested for their ability to kill HIV-infected cells. In one study, sCD4 was coupled to the deglycosylated A chain of ricin which inactivates ribosomes, therefore inhibiting protein synthesis and killing the cell (27). This fusion protein was reported to specifically lyse cells infected with five different isolates of HIV, but was nontoxic to uninfected cells. In another study, the V1V2 domains of CD4 were coupled to domains II and III of Pseudomonas exotoxin A (28). This fusion protein was reported to specifically bind and inhibit protein synthesis in cells expressing the HIV envelope glycoprotein gp120 (25).

We have now discovered that a specific CD4-gamma1 chimeric heavy chain homodimer provides advantages relative to those CD4-IgG1 heavy chain homodimers which have been describ d more than one year ago. Specifically, we have constructed a CD4-gamma1 chimeric heavy chain homodimer which contains the V1V2 domains of CD4 and which is efficiently assembled intracellularly and efficiently secreted from mammalian cells as a homodimer, enabling high recovery and purification from the medium of cells expressing this chimeric heavy chain homodimer. To construct this homodimer, we have used the entire hinge, CH2 and CH3 domains from a human gamma1 heavy chain, which results in a chimeric molecule containing the constant domains of a human IgG1 molecule responsible for dimerization and efficient secretion. This is in contrast to the heavy chain dimers described by Capon and Gregory (20) which include the CH1 domain in the CD4-IgG1 heavy chain dimer, resulting in poor

Summary of the Inv ntion

This invention provides an expression vector encoding a CD4-gamma1 chimeric heavy chain homodimer. This invention also provides an expression vector encoding the heavy chains of a CD4-IgG1 chimeric heterotetramer. Finally, this invention provides an expression vector encoding the light chains of a CD4-IgG1 chimeric heterotetramer.

letter code). The protein d mains are indicated above the sequences by arr ws.

Figure 5: DNA and predicted protein sequence of a CD4-kappa chimeric light chain of the CD4-IgG1 chimeric heterotetramer. The numbers at the end of each line indicate the nucleotide positions. The numbers above each line indicate the amino acid positions (given in single letter code). The protein domains are indicated above th sequences by arrows.

Figure 6: Secretion of CD4-gamma1 chimeric heavy chain homodimer from transfected cells. Cos-M5 cells were mock transfected, transfected with CD4-IgG1-pcDNA1 DNA, r transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post-transfection, the cells were radiolabelled with ^{35}S-methionine. Radiolabelled medium was precipitated with Protein-A sepharose beads. The precipitated proteins were analyzed by SDS-PAGE under reducing or non-reducing conditions and were visualized by fluorography. Lane M, medium from mock transfected cells; Lane 1, medium from cells transfected with CD4-IgG1-pcDNA1 DNA; Lane 2, medium from cells transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA.

Figure 7: Precipitation of HIV-1 gp120 with CD4-gamma1 chimeric heavy chain homodimer. Cos-M5 cells were mock transfected, transfected with the CD4-IgG1-pcDNA1, or transfected with CD4-gamma2 chimeric heavy chain mammalian expression vector DNA. At 48-72 hours post transfection, unlabelled aliquots of medium were incubated with an aliquot of ^{35}S-methionine-labelled gp120. The complexes were precipitated with Protein A-sepharose beads. The precipitates were then analyzed by SDS-PAGE followed by fluorography. Lane M, medium from mock transfected cells;

Figure 11: Purification of CD4-gamma1 chimeric heavy chain h modimer. Stable CHO cells c nstitutively secr ting CD4-gamma1 chimeric heavy chain homodimer were grown in roller bottles. Conditioned medium was passed over a Protein A-sepharose column and bound material was eluted from the column (see Figure 8). The peak fractions were then pooled and passed over an S-sepharose column. After extensive washes, the CD4-gamma1 chimeric heavy chain homodimer was eluted with 50mM BES pH 7.0, 500mM NaCl. The peak fractions were identified by SDS-PAGE followed by silver staining and pooled. The purified protein was then analyzed by SDS-PAGE under non-reducing and reducing conditions followed by silver staining. Lane 1: approximately 1.5μg protein run under non-reducing conditions, Lane 2: approximately 1.5μg protein run under reducing conditions.

Figure 12: Secretion of CD4-IgG1 chimeric heterotetramer from stably transfected cells. CHO cells stably expressing both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains were radiolabelled with ^{35}S-methionine and cysteine. Radiolabelled medium was precipitated with Protein-A sepharose beads. (A) The precipitated proteins were analyzed by SDS-PAGE under non-reducing conditions, and were visualized by fluorography. Lane 1: medium from untransfected CHO cells, Lane 2: medium from cells stably expressing both the CD4-IgG1 chimeric heavy chains, and CD4-kappa chimeric light chains. (B) An identical sample to that run in lane 2 from (A) was run on SDS-PAGE under non-reducing conditions. The lane from this SDS-PAGE gel was excised and the proteins reduced by incubation of the gel slice for 45 minutes at 4°C in equilibration buffer (62.5 mM TrisHCl pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol). After incubation of the gel slice under reducing conditions, the proteins contained within the gel were analyzed by SDS-PAGE and visualized by fluorography.

selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or resistence to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama. (32)

Thus, the invention further provides a method of producing a CD4-gamma1 chimeric heavy chain homodimer. This method comprises

a) transfecting a mammalian cell with an expression vector for producing the CD4-gamma1 chimeric heavy chain homodimer;
b) culturing the resulting transfected mammalian cell under conditions such that CD4-gamma1 chimeric heavy chain homodimer is produced; and
c) recovering the CD4-gamma1 chimeric heavy chain homodimer so produced.

Once the vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an appropriate mammalian cell host. Various techniques may be employed such as protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity. Expression of the gene(s) results in production of the fusion protein which corresponds to one chain of the CD4-gamma1 chimeric heavy

administering th homodimer are well known in the art and includ , merely by way of example, subcutaneous, intramuscular and intravascular injection, alone or in combination with other agents such as AZT or DDI.

Further provided is a method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of the CD4-gamma1 chimeric heavy chain homodimer in an amount which is effective to block the spread of HIV infection.

For example, the homodimer may be administered to patients having HIV infection at a dosage capable of maintaining a concentration of greater than about 100 ng of CD4-gamma1 chimeric heavy chain homodimer/ml plasma. For CD4-gamma1 chimeric heavy chain homodimer variants having different molecular weights, about 2 picomoles of soluble receptor per ml of plasma, an amount for example, sufficient to establish a stoichiometric equivalence with native (membrane bound) and soluble receptor is administered. Typically, the dosage of soluble CD4 is about 100 μg/kg of patient weight/day.

The foregoing method may be used to help prevent the spread of the HIV virus within the body of a HIV infected patient. Additionally, CD4-gamma1 chimeric heavy chain homodimer may be administered as a prophylactic measure to render a subject's blood less susceptible to the spread of the HIV virus. Such prophylactic administration includes administration both prior to HIV contact or shortly thereafter, or both.

A pharmaceutical composition which comprises the CD4-gamma1 chimeric heavy chain homodimer of this invention in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acc ptable carrier is further provided.

detectabl marker, one may identify cells which are infected with HIV. Examples of conventional detectable markers includes radioisotopes such as I125, chromophores, and fluorophores.

Thus, the chimeric heavy chain homodimer of the invention may be used in an assay for HIV or SIV viral infection in a biological sample by contacting a sample derived from an animal suspected of having an HIV or SIV infection, with the homodimer of the invention, and detecting whether a complex forms with gp120, either alone or on the surface of an HIV-infected cell. For this purpose the homodimer may be labeled with a detectable marker or may be unlabeled and then be detected with another reagent which is detectably labeled and is specifically directed to the homodimer or to a complex between it and gp120.

For example, a biological sample may be treated with nitrocellulose, or another solid support which is capable of immobilizing cells, cell particles or soluble protein. The support may then be washed with suitable buffers followed by treatment with the chimeric heavy chain homodimer which may be detectably labeled. The solid phase support may then be washed with buffer a second time to remove unbound fusion protein and the labeled homodimer detected.

In carrying out the assay the following steps may be employed.

a) contacting a sample suspected of containing gp120 with a solid support to effect immobilization of gp120, or cells which express gp120 on their surface;

b) contacting said solid support with the det ctably labeled chimeric heavy chain homodimer of the invention;

a) contacting a mixture obtained by contacting a sample suspected f containing gp120 with a homodimer of this invention, and the Fc portion of an immunoglobulin chain, with an Fc binding molecule, such as an antibody, protein A, or protein G, which is immobilized on a solid phase support and is specific for the homodimer, to obtain a gp120-homodimer immobilized antibody complex,

b) washing the solid phase support obtained in step (a) to remove unbound homodimer; and

c) detecting the homodimer.

Of course, the specific concentrations of unlabeled or detectably labeled homodimer and gp120, the temperature and time of incubation, as well as other assay conditions, may be varied depending on various factors including the concentration of gp120 in the sample, the nature of the sample, and the like. Those skilled in the art are readily able to determine operative and optimal assay conditions for each determination.

Also provided is an enzyme-linked immunoadsorbent assay (ELISA) to detect and quantify soluble CD4 (sCD4) or CD4 chimeric proteins. In carrying out the assay, the process comprises:

a) contacting a sample containing sCD4 with a solid support to immobilize soluble sCD4;

b) contacting said solid support with the detectably labeled monoclonal antibody OKT4a alone, or with a sample containing sCD4 or CD4 chimeric proteins and OKT4a;

c) incubating said detectably labeled OKT4a containing media for sufficient time to allow for binding to immobilized SCD4;

Methods of cotransfecting mammalian cells are well known in th art and include thos discussed her inabove. Similarly, expression vectors encoding light chains are well known in the art.

The invention additionally provides a method of producing a CD4-IgG1 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector for producing the light chains of a CD4-IgG1 chimeric heterotetramer and with an expressi n vector encoding an IgG1 heavy chain;

b) culturing the resulting cotransfected mammalian cell under conditions such that a CD4-IgG1 chimeric hetero-tetramer is produced; and

c) recovering the CD4-IgG1 chimeric heterotetramer so produced.

Further the invention provides a method of producing an CD4-IgG1 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector for producing the heavy chains of a CD4-IgG1 chimeric heterotetramer and an expression vector for producing the light chains of a CD4-IgG1 chimeric heterotetramer;

b) culturing the resulting cotransfected mammalian cell under conditions such that the CD4-IgG1 chimeric heterotetramer is produced; and

c) recovering the CD4-IgG1 chimeric heterotetramer so produced.

The invention also includes a method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are ncoded by th

heterotetramer, the light chains of which ar encoded by th expr ssion vector designated CD4-kLC-pRcCMV, or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above described expression vectors, in an amount effective to inhibit HIV infection of a CD4+ cell, and a pharmaceutically acceptable carrier.

Further provided by the invention is a composition of matter comprising either a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV, or a CD4-IgG1 chimeric heterotetramer, both the heavy and the light chains of which are encoded by the above described expression vectors, and a toxin linked thereto.

In one embodiment of the invention, the toxin is the deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, Diphtheria toxin, or a non-peptidyl cytotoxin.

The invention further provides a diagnostic reagent either comprising a CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector designated CD4-IgG1HC-pRcCMV; a CD4-IgG1 chimeric heterotetramer the light chains of which are encoded by the expression vector designated CD4-kLC-pRcCMV; or a CD4-IgG1 chimeric heterotetramer both the heavy and the light chains of which are encoded by both of those expression vectors, and a detectable marker linked thereto. Examples of suitable detectable markers are radioisotopes, chromophores or fluorophores.

Experimental Details

A. **Materials and Methods**

1. Construction of CD4-gamma1 chimeric heavy chain gene encoding CD4-gamma1 chimeric heavy chain homodimer:

The human CD4 cDNA was excised from the plasmid pSP6T4 (4) as an EcoR1/Stu1 restriction fragment. The 0.70 kilobase fragment was isolated and cloned into EcoR1/Sma1 digested M13mp18. This intermediate vector (M13mp18(CD4)) was then isolated, linearized with Pst1, purified, and treated with Bacterial Alkaline Phosphatase (BAP). The 2.0 Kb Pst1/Pst1 fragment from the plasmid pBr gamma1 containing the human gamma1 heavy chain gene (30), (containing the hinge, CH2, and CH3 exons) was isolated and cloned into the BAP-treated M13mp18/CD4 vector. Resulting recombinants were then screened for the correct orientation of the Pst1 fragment (with respect to the CD4 sequence) to obtain a vector which contains in tandem CD4(EcoR1/Stu1) - gamma1(Pst1/Pst1). To obtain a CD4-gamma1 chimeric heavy chain gene, oligonucleotide-mediated site-directed mutagenesis was performed to juxtapose the CD4 and gamma1 heavy chain DNA sequences, ligating the CD4 sequence in frame to the hinge exon. The resulting chimeric DNA molecule encodes a protein containing the V1V2 domains of CD4 followed by the hinge, CH2, and CH3 domains of gamma1 heavy chain (Figure 1A). Mutagenesis was performed on single-stranded DNA isolated from recombinant phage from transformed TG1 cells (Amersham). Briefly, template DNA was annealed with a 34-mer oligonucleotide (5'-GTCACAAGATTTGGGCTCGAAAGCTAGCACCACG-3'), containing sequences which join the last codon encoding Phe(179) fr m V1V2 of CD4 to the first cod n of the hinge for IgG1 (encoding Glu) (Figures 1A and 3). After second strand synthesis, doubl stranded DNA was transformed into

CosM5 cells grown in DMEM containing 10% f tal calf serum were split to 75% conflu nce. On the following day, th cells were transfected for 16-20 hours with 10 micrograms of CsCl-purified plasmid CD4IgG1-pcDNA1 DNA by the standard CaPO(4) precipitation technique. After transfection, fresh medium was added to the cells. Analysis of the products synthesized 48-72 hours post-transfection was performed by radiolabelling of transfectants with ^{35}S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions (Figure 6). In addition, analysis of media and cell lysates was performed 48-72 hours post-transfection by standard Western blotting procedures.

b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) were transfected with 20 micrograms of CsCl purified DNA in a 1000:1 molar ratio of CD4IgG1-pcDNA1:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may also be used. Approximately 3-5 days post-transfection, cells wer placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). Approximately 10-15 days post-selection, individual cell clones were picked and analyzed for stable expression of CD4-gamma1 chimeric heavy chain homodimer by several screening techniques, such as ELISA and precipitation with Protein A-sepharose beads followed by SDS-PAGE under reducing and non-reducing conditions. Clones expressing the highest levels were subjected to successive rounds of amplification of the newly introduced DNA sequences in increasing concentrations of methotrexate. Stable CHO cell lines were thus generated which secrete between 10-100 micrograms/milliliter of CD4-gamma1 chimeric heavy chain homodimer.

5. Demonstration of binding of CD4-gamma1 chimeric heavy chain hom dimer to the HIV envelope glycoprotein gp120:

CosM5 transfectants expressing CD4-gamma1 chimeric heavy chain homodimer were incubated for 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium was then collected and used to precipitate ^{35}S-methionine-radiolabelled HIV gp120. After incubation of CD4-gamma1 chimeric heavy chain homodimer containing medium with ^{35}S-methionine-labelled gp120, the complexes were adsorbed to Protein A-sepharose. Protein A-sepharose complexes were recovered by centrifugation, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography (Figure 7). Alternatively, aliquots of purified CD4-gamma1 chimeric heavy chain homodimer from CHO cells were also used to precipitate ^{35}S-radiolabelled gp120 using the same procedure.

6. Determination of plasma half-life and placental transfer of CD4-gamma1 chimeric heavy chain homodimer:

Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously or intramuscularly with purified CD4-gamma1 chimeric heavy chain homodimer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-gamma1 chimeric heavy chain homodimer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-gamma1 chimeric heavy chain homodimer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-gamma1 chimeric heavy chain homodimer in the mother's serum as well as in the cord blood and serum of the newborn indicates the

used (31). After incubation for one hour at 4 d gre s C lsius, the ' psonized' virus is added to th cell types described in the paragraph above. At various time points after infection, the media is harvested and assayed for viral reverse transcriptase activity to determine the degree of viral infection. As controls, sCD4, OKT4a or Leu3a are included during the infection of the cells. In addition, various dilutions of the CD4-gamma1 chimeric heavy chain homodimer and appropriate controls are first incubated with the cells at 4 degress Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

8. HIV binding assay:

Binding of HIV was performed as previously described (38, 39). Briefly, concentrated HIV-1 preparations were incubated with various dilutions of sCD4, CD4-gamma1, or CD4-gamma2, for 30 minutes and then added to 5 x 10^5 CEM cells. Bound virus was detected by indirect immunofluorescence and cytofluorography as previously described (39).

9. Neutralization assay:

The microculture assay for productive viral replication was as previously described (38, 40). Briefly dilutions of sCD4, CD4-gamma1, or CD4-gamma2 were incubated for 30 minutes with 100 $TCID_{50}$ HIV-1 at room temperature. The mixtures were added to PHA-stimulated lymphocytes and incubated at 37°C overnight. The cells were then washed and plated in microculture at 1 x 10^5 cells/culture; and 10 cultures per dilution and monitored for reproductive viral replication by detection of HIV antigen in culture supernates 8 and 12 days later.

to express CD4-IgG1 chimeric heavy chains or CD4-kappa chimeric light chains (either alone or in combination) containing only the V1 domain of CD4 were unsuccessful.

2. Construction of CD4-IgG1 chimeric heavy chain expression vector and CD4-kappa chimeric light chain expression vector for production of CD4-IgG1 chimeric heterotetramers.

a. Construction of CD4-IgG1 chimeric heavy chain mammalian expression vector.

The human CD4 cDNA sequence is excised from the plasmid pSP6T4 (4) as an EcoR1/Stu1 restriction fragment. The 0.70 kilobase fragment is isolated and cloned into EcoR1/Sma1-digested M13mp18. The resulting vector (M13mp18(CD4)) is then isolated and digested with BamH1. The BamH1 sites of the M13mp18(CD4) are made flush ended with the Klenow fragment of DNA polymerase 1. After heat inactivation of the polymerase for 15 minutes at 65 degrees Celsius, the linearized M13mp18(CD4) vector is then digested with Pst1 and purified.

In order to excise a fragment containing the CH1 exon of th human gamma1 heavy chain gene, the plasmid pBr gamma1 (30) is digested with SacII, and the SacII sites are then made flush using T4 DNA polymerase. After heat inactivation of the polymerase, the fragment is then digested with Pst1. The resulting SacII(flush)-Pst1 fragment containing the CH1 exon is then purified and ligated to the M13mp18(CD4) vector described in the above paragraph. After transformation of competent TG1 cells, the resulting recombinants are screened by restriction analysis for the presence of both CD4 and CH1 sequences which contain in tandem CD4 (EcoR1/Stu1) - CH1 (SacII(flush)/Pst1). Oligonucleotide-mediated sit -directed

nd d DNA is then ligated overnight at 15 d gr es Celsius with T4 DNA ligase to a 100-fold molar excess of HindIII linkers. After heat inactivation of T4 DNA ligase for 15 minutes at 70 degrees Celsius, the HindIII-linkered DNA is extensively digested with HindIII to liberate a fragment containing the CD4-IgG1 chimeric heavy chain gene. This HindIII fragment is then purified and ligated to the expression vector pcDNA-1 (Invitrogen), which was previously digested with HindIII and BAP treated. The resulting plasmid is then transformed into MC1061/P3 cells. Plasmid DNA is isolated from recombinant clones, and verification of the presence of the HindIII insert and orientation of th insert with respect to the cytomegalovirus (CMV) promoter in the plasmid is made by restriction analysis. The resulting mammalian expression plasmid which encodes a CD4-IgG1 chimeric heavy chain is designated CD4-IgG1HC-pRcCMV.

b. Construction of a CD4-kappa chimeric light chain mammalian expression vector:

The human kappa light chain constant region is excised from the plasmid pCNkappa light as an Mse1 fragment. The purified Mse1 fragment is then made flush ended using the Klenow fragment of DNA polymerase 1. M13mp18 Rf is then linearized with HincII, and the flush ended Mse1 kappa light chain fragment is ligated to M13mp18 at the flush ended HincII site in the vector. After transformation of TG1 cells, the recombinants are confirmed for the presence of the insert and the correct orientation within the vector by restriction analysis. Rf is purified from infected TG1 cells and digested with EcoR1 and Sma1. The purified vector containing the kappa light chain constant region is then ligated to the EcoR1/Stu1 fragment of the human CD4 cDNA described above. The resulting recombinants are then verified for the presence and orientation of both inserts

r sulting mammalian expression plasmid which encod s a CD4-kappa chimeric light chain is designated CD4-kLC-pRcCMV.

3. Co-expression of CD4-IgG1HC-pRcCMV and CD4-kLC-pRcCMV in mammalian cells to produce CD4-IgG1 chimeric heterotetramer.

a. Transient expression.

CosM5 cells grown in DMEM containing 10% fetal calf serum are split to 75% confluence. On the following day, the cells are transfected for 16-20 hours with 5 micrograms of CsCl purified CD4-IgG1HC-pRcCMV DNA and 5 micrograms of CsCl-purified CD4-kLC-pRcCMV plasmid DNA by the standard CaPO(4) precipitation technique. After transfection, fresh medium is added to the cells. Analysis of the products synthesized 48-72 hours post-transfection is performed by radiolabelling of transfectants with ^{35}S-methionine for 12-18 hours followed by precipitation of media and cell lysates using anti-CD4 antibodies or by incubation with Protein A-sepharose beads alone followed by SDS-PAGE under reducing or non-reducing conditions. In addition, analysis of media and cell lysates is performed 48-72 hours post-transfection by standard Western blotting procedures.

b. Stable expression.

Dhfr-Chinese hamster ovary cells (CHO) are transfected with 20 micrograms of CsCl purified DNA in a ratio of 1000:1000:1 CD4-IgG1HC-pRcCMV:CD4-kLC-pRcCMV:p410 (p410 is an expression plasmid containing the dhfr gene), although other ratios may also be used. At approximately 3-5 days post-transfection, cells are placed in selective medium (nucleoside-free alpha MEM containing 10% dialyzed fetal calf serum). At approximately 10-15 days post-selection, individual cell

CosM5 transfectants xpressing CD4-IgG1 chim ric heterotetramers are incubated f r 72 hours in DMEM containing 10% IgG-free fetal calf serum. Unlabelled medium is then collected and used to precipitate ^{35}S-methionine-radiolabelled HIV gp120. After incubation of CD4-IgG1 chimeric heterotetramer containing medium with ^{35}S-methionine-labelled gp120, the complexes are adsorbed to Protein A-sepharose. Protein A-sepharose complexes are recovered by centrifugation, and the precipitates are analyzed by SDS-PAGE followed by fluorography. Alternatively, aliquots of purified CD4-IgG1 chimeric heterotetramers from CHO cells are also used to precipitate ^{35}S-radiolabelled gp120 using the same procedure.

6. Determination of plasma half-life and placental transfer of CD4-IgG1 chimeric heterotetramer:

Determination of the plasma half-life and placental transfer are performed by well established techniques. Briefly, rabbits or monkeys are injected intravenously r intramuscularly with purified CD4-IgG1 chimeric heterotetramer. At various time points post-injection, plasma samples are taken, and the quantity of the CD4-IgG1 chimeric heterotetramer present in the serum is measured by ELISA. In addition, pregnant monkeys are also injected either IV or IM with CD4-IgG1 chimeric heterotetramer and the concentration determined in the cord blood and the serum of the newborn monkey. Determination and comparison of the quantity of the CD4-IgG1 chimeric heterotetramer in the mother's serum as well as in the cord blood and serum of the newborn indicates the relative rate of transport across the placenta of these molecules.

7. Determination of FcR binding and macrophage infectivity of CD4-IgG1 chimeric heterotetramer:

included during the infection of th cells. In addition, various dilutions of the CD4-IgG1 chimeric heterotetramer and appropriate controls are incubated first with the cells at 4 degress Celsius to allow binding. HIV is then added and infection assayed by viral reverse transcriptase activity.

B. Results:

A CD4-gamma1 chimeric heavy chain gene encoding a CD4-gamma1 chimeric heavy chain homodimer was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA (4) to the hinge exon of the human gamma1 heavy chain gene (30) (Figure 1A). The resulting recombinant DNA molecule (designated CD4-IgG1-Rf) encodes the signal sequence and two amino-terminal immunoglobulin-like domains of the CD4 protein (the first 179 amino acids of mature CD4) followed by the hinge (15 amino acids), CH2 (110 amino acids), and CH3 (107 amino acids) regions of the gamma1 heavy chain protein (Figure 3). This recombinant DNA molecule also contains two introns present within the gamma1 heavy chain gene: between the H and CH2 domains, and between the CH2 and CH3 domains. This CD4-gamma1 chimeric gene was designed to encode a CD4-gamma1 chimeric heavy chain homodimer which specifically lacks the CH1 domain of the gamma1 heavy chain. Expression of the CH1 domain without accompanying light chains prevents efficient heavy chain secretion from mammalian cells (25).

In the CD4-gamma1 chimeric heavy chain homodimer, the hing region of one chain contains three cysteine residues, affording the potential of three interchain disulfide bonds (Figure 1B). In contrast, naturally-occurring human IgG1 contains two interchain disulfide bonds between the gamma1 heavy chains; the amino-terminal cystein in the gamma1 hinge region is disulfide bonded to the final cysteine in the light chain constant regi n, while the two remaining

Mr of approximately 94 kilodaltons. Taken together, these results demonstrate that th CD4-gamma1 chimeric heavy chain is produced and secreted as a homodimer of the predicted molecular weight.

The above results demonstrate that the Fc portion of CD4-gamma1 chimeric heavy chain homodimer, encoded by the constant regions of the gamma1 heavy chain gene, binds Protein A and is therefore functionally active. In order to determine if the CD4 portion is functionally intact, CD4-gamma1 chimeric heavy chain homodimers were assayed for their ability to bind to the HIV exterior envelop glycoprotein, gp120 (Figure 7). Unlabelled medium from CosM5 cells transfected with CD4-IgG1-pcDNA1 DNA was incubated with ^{35}S-methionine-labelled gp120. CD4-gamma1 chimeric heavy chain homodimer/gp120 complexes were precipitated by incubation with Protein A-sepharose beads, and the precipitates were analyzed by SDS-PAGE under reducing conditions followed by fluorography. These results demonstrate that the CD4-gamma1 chimeric heavy chain homodimer efficiently recognizes HIV gp120 and binds with high affinity. These observations, taken together with the results described in the above paragraph, demonstrate that CD4-gamma1 chimeric heavy chain homodimer contains functionally active regions of both CD4 and gamma1 heavy chain.

In order to stably produce large quantities of the CD4-gamma1 chimeric heavy chain homodimers, the CD4-IgG1-pcDNA1 vector was cotransfected with the plasmid p410 (encoding the enzyme dihydrofolate reductase (dhfr)) into dhfr-Chinese Hamster Ovary (CHO) cells. Approximately two weeks post-transfection, individual clones growing in nucleoside free alpha MEM and 10% dialyzed fetal calf serum (and therefore dhfr+) were isolated and analyzed for co-expression of CD4-gamma1 chimeric heavy chain homodimers by precipitation and

neutralization of infectivity of a fixed HIV inoculum (Figure 10). In this later assay, approximately 10-25 μg/ml of CD4-gamma1 as well as sCD4 were required to prevent 50% of the cultures from becoming infected by HIV.

Further purification of CD4-gamma1 heavy chain homodimer was achieved using ion-exchange chromatography. The peak fraction from the protein A-sepharose column was applied to a 10ml S-sepharose fast flow column preequilibrated with 50mM BES pH 7.0, at a flow rate of 120ml/hr. After application of the sample, the column was extensively washed with 50mM BES pH 7.0 with increasing salt concentration (see materials and methods). A single band of CD4-gamma1 heavy chain homodimer was specifically eluted from the column in 50mM BES pH 7.0 containing 500mM NaCl. These peak fractions were pooled and analyzed by SDS-PAGE and silver staining under non-reducing conditions (Figure 11, lane 1), and reducing conditions (Figure 11, lane 2). When the purified CD4-gamma1 chimeric heavy chain homodimer was run on SDS-PAGE under reducing conditions, a doublet was observed which appeared to be due to differences in glycosylation of the CD4-gamma1 chimeric heavy chain homodimer (data not shown).

A CD4-IgG1HC chimeric heavy chain gene encoding a CD4-IgG1 chimeric heavy chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the CH1 exon of the human IgG1 heavy chain gene (Figure 2A). In addition, a CD4-kappa chimeric light chain gene encoding a CD4-kappa light chain was generated by ligating the leader-V1-V2 segment of the human CD4 cDNA to the constant domain of the kappa light chain gene (Figure 2A). These CD4-IgG1 chimeric heavy chain genes and CD4-kappa chimeric light chain genes were designed to encode a CD4-IgG1 chimeric heterotetramer, in which the CD4-IgG1 heavy chain contains a CH1 domain for efficient association with kappa light chains.

which are consistent with the relative pr dicted molecular masses of the CD4-IgG1 chimeric heavy chains, and CD4-kappa chimeric light chains respectively (data not shown). Further characterization has shown that the protein migrating at 210 kilodaltons on SDS-PAGE under non-reducing conditions contains both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains which are covalently associated, while the protein migrating at 140 kilodaltons on SDS-PAGE under non-reducing conditions contains only CD4-IgG1 chimeric heavy chains (Figure 12B). These data ar consistent with the predicted molecular weight for the 210 kilodalton protein being comprised of 2 CD4-IgG1 chimeric heavy chains and 2 CD4-kappa chimeric light chains, covalently associated to form a molecule with the structure H_2L_2 (H=heavy chain, L=light chain). Furthermore, the 140 kilodalton protein seen on SDS-PAGE under non-reducing conditions is consistent with the predicted molecular weight of a CD4-IgG1 chimeric homodimer with the structure H_2. Taken together, these results indicate that a CHO cell line which expresses both CD4-IgG1 chimeric heavy chains and CD4-kappa chimeric light chains is able to efficiently assemble and secrete CD4-IgG1 chimeric heterotetramers.

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What is claimed is :

1. An expression vector encoding a CD4-gamma1 chimeric heavy chain homodimer designated CD4-IgG1-pcDNA1 (ATCC No. 40951).

2. A CD4-gamma1 chimeric heavy chain homodimer encoded by the expression vector of claim 1.

3. A method of producing a CD4-gamma1 chimeric heavy chain homodimer which comprises:

a) transfecting a mammalian cell with the expression vector of claim 1;

b) culturing the resulting transfected mammalian cell under conditions such that chimeric heavy chain homodimer is produced; and

c) recovering the chimeric heavy chain homodimer so produced.

4. A method of claim 3, wherein the mammalian cell is a COS cell, CHO cell or myeloma cell.

5. A method of inhibiting HIV infection of a CD4+ cell which comprises treating the CD4+ cell with an amount of the CD4-gamma1 chimeric heavy chain homodimer of claim 2 effective to inhibit infection of the cell.

6. A method of preventing a subject from being infected with HIV which comprises administering to the subject an amount of the CD4-gamma1 chimeric heavy chain homodimer of claim 2 effective to prevent the subject from being infected with HIV.

15. A CD4-IgG1 chimeric heterotetramer, the heavy chains of which are encoded by the expression vector of claim 13.

16. A CD4-IgG1 chimeric heterotetramer, the light chains of which are encoded by the expression vector of claim 14.

17. A CD4-IgG1 chimeric heterotetramer the heavy and the light chains of which are encoded by the expression vectors of claims 13 and 14, respectively.

18. A method of producing a CD4-IgG1 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with th expression vector of claim 13 and an expression vector encoding a light chain;

b) culturing the resulting cotransfected mammalian cell under conditions such that th CD4-IgG1 chimeric heterotetramer is produced; and

c) recovering the CD4-IgG1 chimeric heterotetramer so produced.

19. A method of producing an CD4-IgG1 chimeric heterotetramer which comprises:

a) cotransfecting a mammalian cell with the expression vector of claim 14 and an expression vector encoding an IgG1 heavy chain and;

24. A method of treating a subject infected with HIV so as to block the spread of HIV infection which comprises administering to the subject an amount of CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 effective to block spread of HIV infection.

25. A pharmaceutical composition which comprises the CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 in an amount effective to inhibit HIV infection of a CD4+ cell and a pharmaceutically acceptable carrier.

26. A composition of matter comprising a CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 and a toxin linked thereto.

27. A composition of claim 26, wherein the toxin is th deglycosylated A chain of ricin, domains II or III of Pseudomonas exotoxin A, and Diphtheria toxin.

28. A diagnostic reagent comprising a CD4-IgG1 chimeric heterotetramer of claim 15, 16 or 17 and a detectable marker linked thereto.

29. A diagnostic reagent of claim 28 wherein the detectable marker is a radioisotope, chromophore or fluorophore.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01152

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/387.3, 391.1, 191.3, 391.7, 866; 435/ 69.3, 69.7; 424/85.8, 85.9, 85.91; 436/ 514; 935/12, 15

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[6]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X Y	NATURE, VOLUME 339, ISSUED 04 MAY 1989, TRAUNECKER ET AL, "HIGHLY EFFICIENT NEUTRALIZATION OF HIV WITH RECOMBINANT CD4-IMMUNOGLOBULIN MOLECULES", PAGES 68-70, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
X Y	WO, A, 89/03222 (REINHERZ ET AL) 20 APRIL 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
Y	WO, A, 88/01304 (MADDON ET AL) 25 FEBRUARY 1988, SEE ENTIRE DOCUMENT.	1-4,8-21, 25-29
X Y	EP, A, 0,314,317 (CAPON ET AL) 03 MAY 1989,SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
Y	CELL, VOLUME 42, ISSUED AUGUST 1985, MADDON ET AL, "THE ISOLATION AND NUCLEOTIDE SEQUENCE OF A cDNA ENCODING THE T CELL SURFACE PROTEIN T4: A NEW MEMBER OF THE IMMUNOGLOBULIN GENE FAMILY", PAGES 93-104, SEE ENTIRE DOCUMENT.	1-4,8-21, 25-29
X Y	WO, A, 89/02922, (CAPON ET AL) 06 APRIL 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29
X Y	WO, A, 89/01940, (FISHER ET AL) 09 MARCH 1989, SEE ENTIRE DOCUMENT.	1-4,13-21 8-12,25-29

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
07 MAY 1992	11 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	T. MICHAEL NISBET

Form PCT/ISA/210 (second sheet)(May 1986) 8

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/00, 35/14; C12P 21/06; G01N 33/558; C07K 15/00,13/00; C07H 15/12

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/387.3, 391.1, 391.3, 391.7, 866; 435/ 69.3, 69.7; 424/85.8, 85.9, 85.91; 436/514; 935/12, 15; 536/27

II. FIELDS SEARCHED
Other Documents Searched:

AUTOMATED PATENT SYSTEM, FILE: USPAT; DIALOG ONLINE ONESEARCH; FILE BIOSIS, MEDLINE, BIOTECHNOLOGY ABSTRACTS, EMBASE, WORLD PATENT INDEX
KEYWORDS: CD4, HIV, FUSION OR HETEROLOGOUS()PROTEIN OR PEPTIDE OR POLYPEPTIDE, IMMUNOTOXIN, RICN, DIPTHERIA, TOXIN?

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. CLAIMS 1,3,4,13-14, AND 18-21 DRAWN TO EXPRESSION VECTORS AND METHODS OF USING THOSE VECTORS CATAGORIZED AS A FIRST APPEARING PRODUCT AND A FIRST APPEARING METHOD OF USING THAT PRODUCT.
II. CLAIMS 2,8-12, 15-17, AND 25-29 DRAWN POLYPEPTIDES, PHARMACEUTICALS, IMMUNOTOXINS, AND DIAGNOSTICS FOR THE PROTEINS ENCODED BY THE VECTORS OF GROUP I. THE PROTEINS AND DERIVATIVES THEREOF ARE SECOND APPEARING PRODUCTS.
III. CLAIMS 5 AND 22 ARE A SECOND APPEARING METHOD OF USING THE PROTIENS OF GROUP II FOR INHIBITING HIV INFECTION.
IV. CLAIMS 6 AND 23 ARE A THIRD APPEARING METHOD OF USING THE PROTEINS OF GROUP II FOR PREVENTING HIV INFECTION.
V. CLAIMS 7 AND 24 ARE A FOURTH APPEARING METHOD OF USING THE PROTEINS OF GROUP II FOR TREATING SUBJECTS INFECTED WITH HIV.

Figure 1A

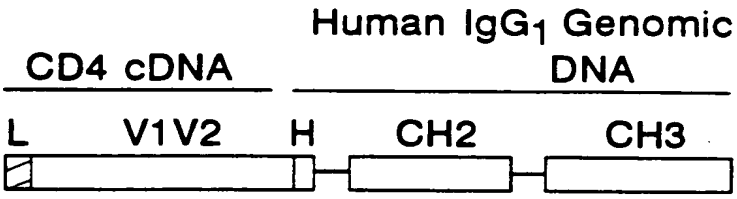

Figure 1B

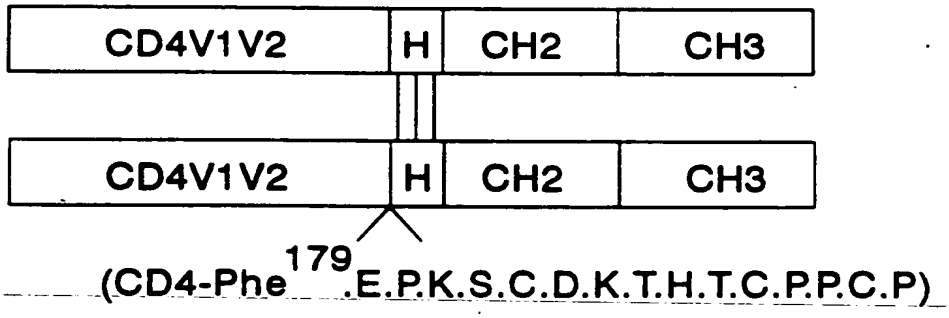

Figure 2A

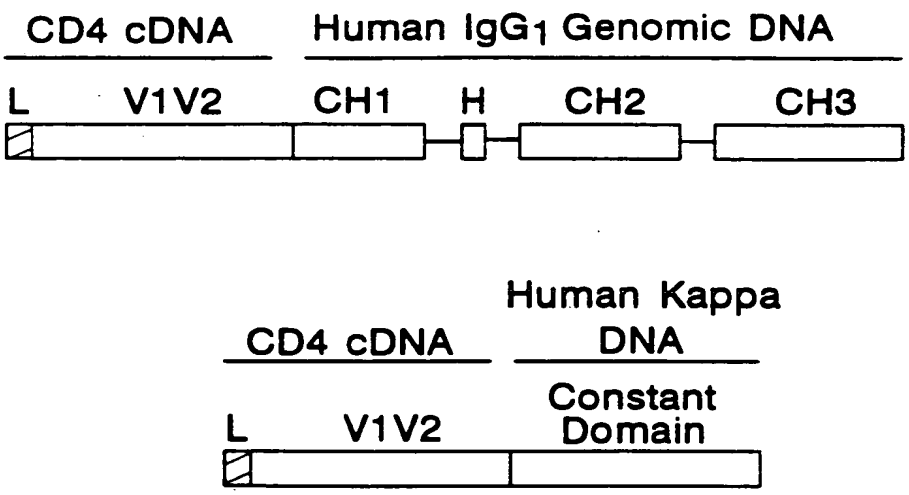

Figure 2B

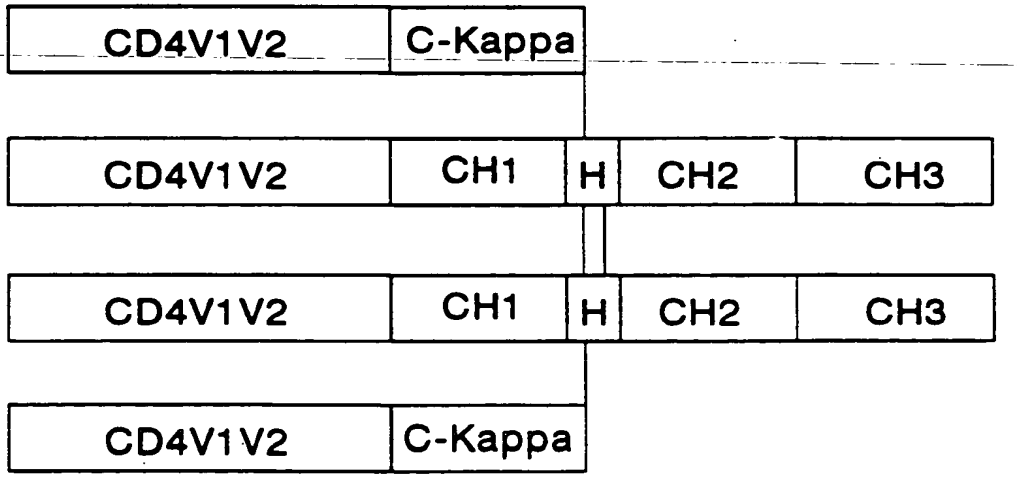

Figure 3A

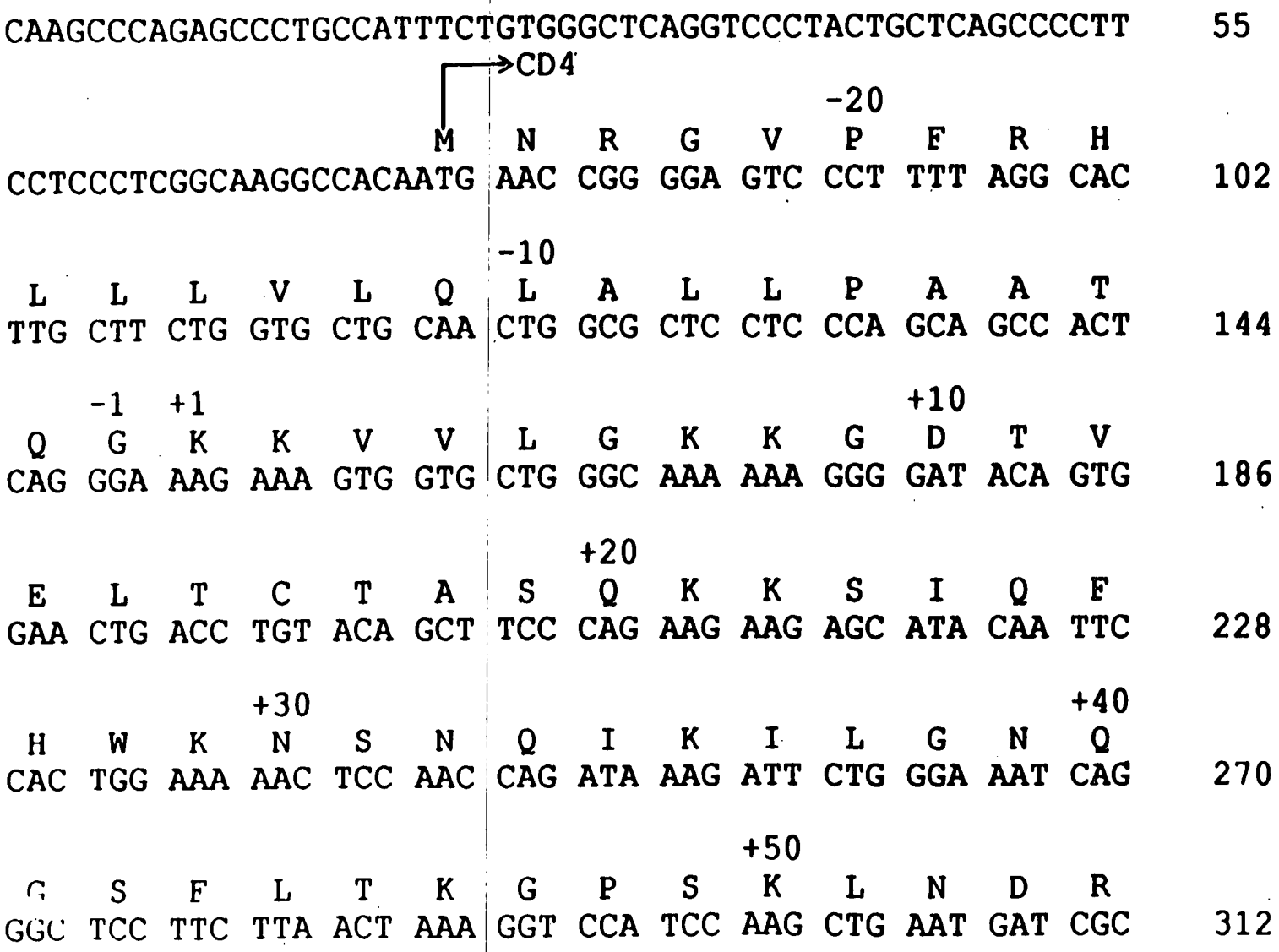
CAAGCCCAGAGCCCTGCCATTTCTGTGGGCTCAGGTCCCTACTGCTCAGCCCCTT 55
CD4
-20
M N R G V P F R H
CCTCCCTCGGCAAGGCCACAATG AAC CGG GGA GTC CCT TTT AGG CAC 102
-10
L L L V L Q L A L L P A A T
TTG CTT CTG GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT 144
-1 +1 +10
Q G K K V V L G K K G D T V
CAG GGA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA GTG 186
+20
E L T C T A S Q K K S I Q F
GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC 228
+30 +40
H W K N S N Q I K I L G N Q
CAC TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG 270
+50
G S F L T K G P S K L N D R
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC 312

Figure 3B

```
                                +60
 A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC    354

    +70                                             +80
 L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC    396

                                    +90
 I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA    438

            +100                                    +110
 V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG    480

                                        +120
 G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT    522

                        +130
 S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA    564
```

Figure 3C

```
  +140                                      +150
 Q   G   G   K   T   L   S   V   S   Q   L   E   L   Q
CAG GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG     606

                          +160
 D   S   G   T   W   T   C   T   V   L   Q   N   Q   K
GAT AGT GGC ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG     648
                                                     ┌→Hinge
            +170                                  +180
 K   V   E   F   K   I   D   I   V   V   L   A   F   E
AAG GTG GAG TTC AAA ATA GAC ATC GTG GTG CTA GCT TTC GAG     690

                                    +190
 P   K   S   C   D   K   T   H   T   C   P   P   C   P
CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA     732

GGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAG     787

TAGCCTGCATCCAGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCT     842
       ┌──→CH2
       │                  +200
       A   P   E   L   L   G   G   P   S   V   F   L
CTTCCTCAGCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC     886
```

Figure 3D

```
                                                                +220
  F   P   P   K   P   K   D   T   L   M   I   S   R   T
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC    928

                                +230
  P   E   V   T   C   V   V   V   D   V   S   H   E   D
CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC    970

                        +240                    +260
  P   E   V   K   F   N   W   Y   V   D   G   V   E   V
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG   1012

    +250                        +270
  H   N   A   K   T   K   P   R   E   E   Q   Y   N   S
CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC   1054

  T   Y   R   V   V   S   V   L   T   V   L   H   Q   D
ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC   1096

    +280                        +290
  W   L   N   G   K   E   Y   K   C   K   V   S   N   K
TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA   1138
```

Figure 3E

```
                                             +300
 A    L    P    A    P    I    E    K    T    I    S    K    A    K
GCC  CTC  CCA  GCC  CCC  ATC  GAG  AAA  ACC  ATC  TCC  AAA  GCC  AAA       1180

GGTGGGACCCGTGGGGTGCGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCC                   1235
                                                  ┌>CH3
                                                  │
                                                  G    Q    P    R
TCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCCTACAGGG CAG  CCC  CGA                1288

    +310                                                 +320
 E    P    Q    V    Y    T    L    P    P    S    R    D    E    L
GAA  CCA  CAG  GTG  TAC  ACC  CTG  CCC  CCA  TCC  CGG  GAT  GAG  CTG       1330

                                   +330
 T    K    N    Q    V    S    L    T    C    L    V    K    G    F
ACC  AAG  AAC  CAG  GTC  AGC  CTG  ACC  TGC  CTG  GTC  AAA  GGC  TTC       1372

               +340                                                +350
 Y    P    S    D    I    A    V    E    W    E    S    N    G    Q
TAT  CCC  AGC  GAC  ATC  GCC  GTG  GAG  TGG  GAG  AGC  AAT  GGG  CAG       1414

                                             +360
 P    E    N    N    Y    K    T    T    P    P    V    L    D    S
CCG  GAG  AAC  AAC  TAC  AAG  ACC  ACG  CCT  CCC  GTG  CTG  GAC  TCC       1456
```

Figure 3F

```
                                +370
D   G   S   F   F   L   Y   S   K   L   T   V   D   K
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG   1498

            +380                            +390
S   R   W   Q   Q   G   N   V   F   S   C   S   V   M
AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG   1540

                        +400
H   E   A   L   H   N   H   Y   T   Q   K   S   L   S
CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC   1582

                    +410
L   S   P   G   K   stop
CTG TCT CCG GGT AAA TGAGTGCGACGGCCGGCAAGCCCCCGCTCCCCGGGC  1632

TCTCGCGGTCGCACGAGGATGCTTGGCACGTACCCCCTGTACATACTTCCCGGGC  1687

GCCCAGCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGA  1742

TGGTTCTTTCCACGGGTCAGGCCGAGTCTGAGGCCTGAGTGGCATGAGGGAGGCA  1797

GAGCGGGTC...                                              1806
```

Figure 4A

```
CAAGCCCAGAGCCCTGCCATTTCTGTGGGCTCAGGTCCCTACTGCTCAGCCCCTT      55
                         ┌──>CD4
                                        -20
                         M   N   R   G   V   P   F   R   H
CCTCCCTCGGCAAGGCCACAATG AAC CGG GGA GTC CCT TTT AGG CAC     102

                            -10
 L   L   L   V   L   Q   L   A   L   L   P   A   A   T
TTG CTT CTG GTG CTG CAA CTG GCG CTC CTC CCA GCA GCC ACT     144

    -1  +1                                  +10
 Q   G   K   K   V   V   L   G   K   K   G   D   T   V
CAG GGA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA GTG     186

                                +20
 E   L   T   C   T   A   S   Q   K   K   S   I   Q   F
GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC     228

            +30                                     +40
 H   W   K   N   S   N   Q   I   K   I   L   G   N   Q
CAC TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG     270

                                        +50
 G   S   F   L   T   K   G   P   S   K   L   N   D   R
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC     312
```

Figure 4B

```
                                                                    +60
A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC   354

                +70                                 +80
L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC   396

                                    +90
I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA   438

                +100                                +110
V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG   480

                                        +120
G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT   522

                        +130
S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA   564
```

Figure 4C

```
     +140                                              +150
 Q    G    G    K    T    L    S    V    S    Q    L    E    L    Q
CAG  GGG  GGG  AAG  ACC  CTC  TCC  GTG  TCT  CAG  CTG  GAG  CTC  CAG    606

                                   +160
 D    S    G    T    W    T    C    T    V    L    Q    N    Q    K
GAT  AGT  GGC  ACC  TGG  ACA  TGC  ACT  GTC  TTG  CAG  AAC  CAG  AAG    648
                                                                 ┌→CH1
               +170                                            +180
 K    V    E    F    K    I    D    I    V    V    L    A    F    A
AAG  GTG  GAG  TTC  AAA  ATA  GAC  ATC  GTG  GTG  CTA  GCT  TTC  GCC    690

                                             +190
 S    T    K    G    P    S    V    F    P    L    A    P    S    S
TCC  ACC  AAG  GGC  CCA  TCG  GTC  TTC  CCC  CTG  GCA  CCC  TCC  TCC    732

                         +200
 K    S    T    S    G    G    T    A    A    L    G    C    L    V
AAG  AGC  ACC  TCT  GGG  GGC  ACA  GCG  GCC  CTG  GGC  TGC  CTG  GTC    774

     +210                                                  +220
 K    D    Y    F    P    E    P    V    T    V    S    W    N    S
AAG  GAC  TAC  TTC  CCC  GAA  CCG  GTG  ACG  GTG  TCG  TGG  AAC  TCA    816

                                   +230
 G    A    L    T    S    G    V    H    T    F    P    A    V    L
GGC  GCC  CTG  ACC  AGC  GGC  GTG  CAC  ACC  TTC  CCG  GCT  GTC  CTA    858
```

Figure 4D

```
                +240                                          +250
 Q   S   S   G   L   Y   S   L   S   S   V   V   T   V
CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG     900

                                    +260
 P   S   S   S   L   G   T   Q   T   Y   I   C   N   V
CCC TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG     942

                        +270
 N   H   K   P   S   N   T   K   V   D   K   K   V
AAT CAC AAG CCC AGC AAC ACC AAG GTG GAC AAG AAA GTTGGTGA    986

GAGGCCAGCACAGGGAGGGAGGGTGTCTGCTGGAAGCAGGCTCAGCGCTCCTGCC    1041

TGGACGCATCCCGGCTATGCAGCCCCAGTCCAGGGCAGCAAGGCAGGCCCCGTCT    1096

GCCTCTTCACCCGGAGCCTCTGCCCGCCCCACTCATGCTCAGGGAGAGGGTCTTC    1151

TGGCTTTTTCCCAGGCTCTGGGCAGGCACAGGCTAGGTGCCCCTAACCCAGGCCC    1206

TGCACACAAAGGGGCAGGTGCTGGGCTCAGACCTGCCAAGAGCCATATCCGGGAG    1261
```

Figure 4E

```
GACCCTGCCCCTGACCTAAGCCCACCCCAAAGGCCAAACTCTCCACTCCCTCAGC   1316
                                                      →Hinge
                                                      E
TCGGACACCTTCTCTCCTCCCAGATTCCAGTAACTCCCAATCTTCTCTCTGCAGAG  1372

    +280                                      +290
 P   K   S   C   D   K   T   H   T   C   P   P   C   P
CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA   1414

GGTAAGCCAGCCCAGGCCTCGCCCTCCAGCTCAAGGCGGGACAGGTGCCCTAGAG   1469

TAGCCTGCATCCAGGGACAGGCCCCAGCCGGGTGCTGACACGTCCACCTCCATCT   1524
            →CH2
                                    +300
            A   P   E   L   L   G   G   P   S   V   F   L
CTTCCTCAGCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC   1568

                        +310
 F   P   P   K   P   K   D   T   L   M   I   S   R   T
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC   1610

    +320                                      +330
 P   E   V   T   C   V   V   V   D   V   S   H   E   D
CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC   1652
```

Figure 4F

```
                                    +340
 P   E   V   K   F   N   W   Y   V   D   G   V   E   V
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG   1694

            +350                                    +360
 H   N   A   K   T   K   P   R   E   E   Q   Y   N   S
CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC   1736

                                +370
 T   Y   R   V   V   S   V   L   T   V   L   H   Q   D
ACG TAC CGG GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC   1778

                    +380
 W   L   N   G   K   E   Y   K   C   K   V   S   N   K
TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA   1820

   +390                                    +400
 A   L   P   A   P   I   E   K   T   I   S   K   A   K
GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA   1862

GGTGGGACCCGTGGGGTGCGAGGGCCACATGGACAGAGGCCGGCTCGGCCCACCC   1917
                                          →CH3
                                          G   Q   P   R
TCTGCCCTGAGAGTGACCGCTGTACCAACCTCTGTCCTACAGGG CAG CCC CGA   1970
```

Figure 4G

```
                                                                +420
E   P   Q   V   Y   T   L   P   P   S   R   D   E   L
GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG   2012
                +410
T   K   N   Q   V   S   L   T   C   L   V   K   G   F
ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC   2054
                        +430
Y   P   S   D   I   A   V   E   W   E   S   N   G   Q
TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG   2096
                    +440
P   E   N   N   Y   K   T   T   P   P   V   L   D   S
CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC   2138
            +450
D   G   S   F   F   L   Y   S   K   L   T   V   D   K
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG   2180
                +470
S   R   W   Q   Q   G   N   V   F   S   C   S   V   M
AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG   2222
                        +480
H   E   A   L   H   N   H   Y   T   Q   K   S   L   S
CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC   2264
                    +490
                +500
```

Figure 4H

```
 L   S   P   G   K  stop
CTG TCT CCG GGT AAA TGAGTGCGACGGCCGGCAAGCCCCGCTCCCCGGGC      2313

TCTCGCGGTCGCACGAGGATGCTTGGCACGTACCCCCTGTACATACTTCCCGGGC      2368

GCCCAGCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCCTGCGAGACTGTGA      2423

TGGTTCTTTCCACGGGTCAGGCCGAGTCTGAGGGCCTGAGTGGCATGAGGGAGGCA     2478

GAGCGGGTC...                                                 2487
```

Figure 5A

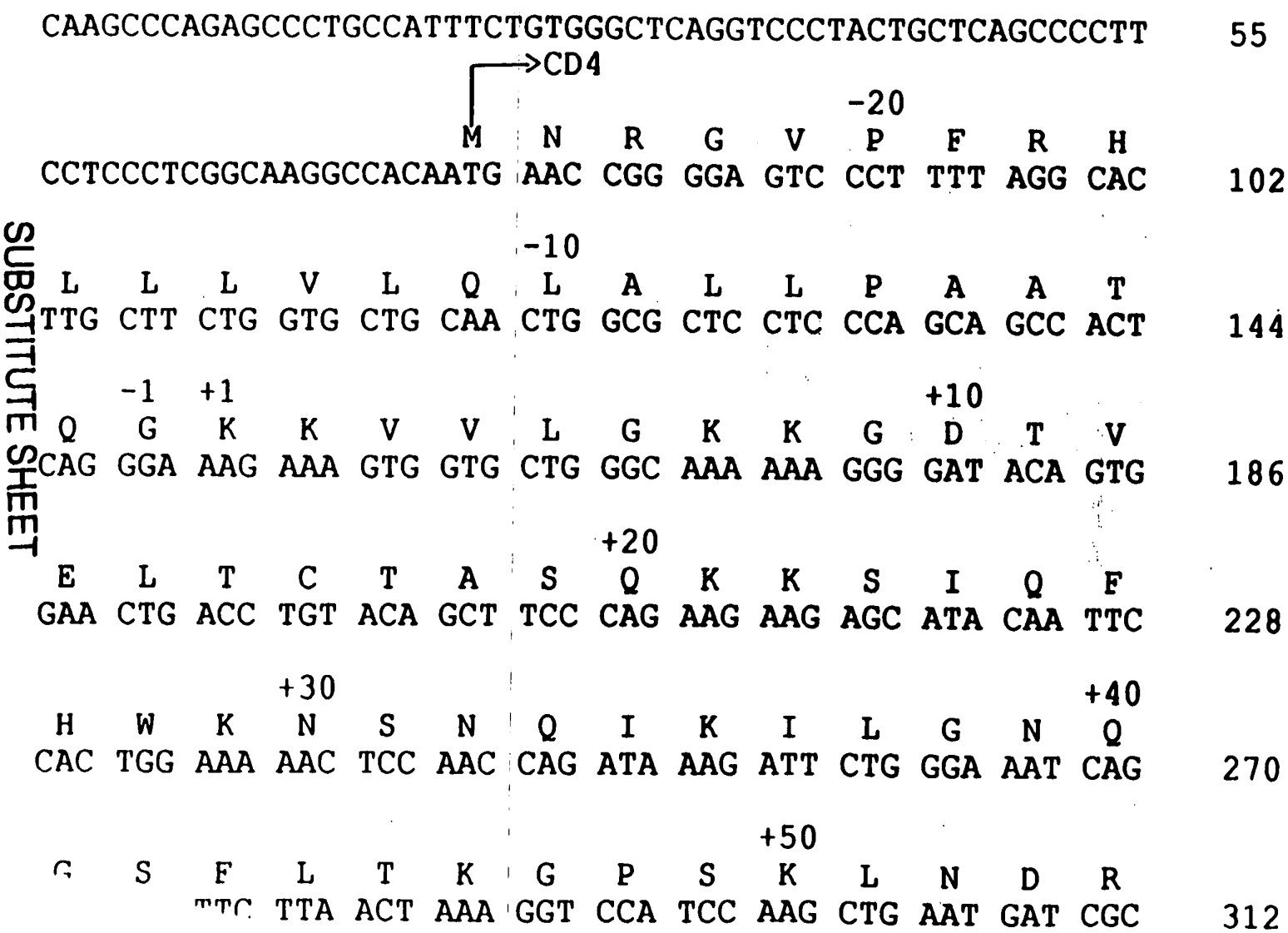

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Figure 5B

```
                         +60
 A   D   S   R   R   S   L   W   D   Q   G   N   F   P
GCT GAC TCA AGA AGA AGC CTT TGG GAC CAA GGA AAC TTC CCC    354

    +70                                     +80
 L   I   I   K   N   L   K   I   E   D   S   D   T   Y
CTG ATC ATC AAG AAT CTT AAG ATA GAA GAC TCA GAT ACT TAC    396

                             +90
 I   C   E   V   E   D   Q   K   E   E   V   Q   L   L
ATC TGT GAA GTG GAG GAC CAG AAG GAG GAG GTG CAA TTG CTA    438

            +100                                    +110
 V   F   G   L   T   A   N   S   D   T   H   L   L   Q
GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC CTG CTT CAG    480

                                    +120
 G   Q   S   L   T   L   T   L   E   S   P   P   G   S
GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT AGT    522

                    +130
 S   P   S   V   Q   C   R   S   P   R   G   K   N   I
AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA    564
```

Figure 5C

```
     +140                                                   +150
 Q    G    G    K    T    L    S    V    S    Q    L    E    L    Q
CAG  GGG  GGG  AAG  ACC  CTC  TCC  GTG  TCT  CAG  CTG  GAG  CTC  CAG    606

                                    +160
 D    S    G    T    W    T    C    T    V    L    Q    N    Q    K
GAT  AGT  GGC  ACC  TGG  ACA  TGC  ACT  GTC  TTG  CAG  AAC  CAG  AAG    648
                                                                 ┌→Ckappa
               +170                                             +180
 K    V    E    F    K    I    D    I    V    V    L    A    F    T
AAG  GTG  GAG  TTC  AAA  ATA  GAC  ATC  GTG  GTG  CTA  GCT  TTC  ACT    690

                                              +190
 V    A    A    P    S    V    F    I    F    P    P    S    D    E
GTG  GCT  GCA  CCA  TCT  GTC  TTC  ATC  TTC  CCG  CCA  TCT  GAT  GAG    732

                         +200
 Q    L    K    S    G    T    A    S    V    V    C    L    L    N
CAG  TTG  AAA  TCT  GGA  ACT  GCC  TCT  GTT  GTG  TGC  CTG  CTG  AAT    774

     +210                                                  +220
 N    F    Y    P    R    E    A    K    V    Q    W    K    V    D
AAC  TTC  TAT  CCC  AGA  GAG  GCC  AAA  GTA  CAG  TGG  AAG  GTG  GAT    716
```

Figure 5D

```
                                +230
  N   A   L   Q   S   G   N   S   Q   E   S   V   T   E
AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC ACA GAG   758

                            +240                    +250
  Q   D   S   K   D   S   T   Y   S   L   S   S   T   L
CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG   900

                                +260
  T   L   S   K   A   D   Y   E   K   H   K   V   Y   A
ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC   942

                            +270
  C   E   V   T   H   Q   G   L   S   S   P   V   T   K
TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG   984

                        +280
  S   F   N   R   G   E   C   stop
AGC TTC AAC AGG GGA GAG TGT TAG AGGGAGAAGTGCCCCCACCTGCTC  1032

CTCAGTTCCAGCCTGACCCCCTCCCATCCTTGGCCTCTGACCCTTTTTCCACAGG  1088

GGACCTACCCCTATTGCGGTCCTCCAAGCTCATCTTTCACCTCACCCCCCTCCTCC  1144

TCCTT
```

Figure 6

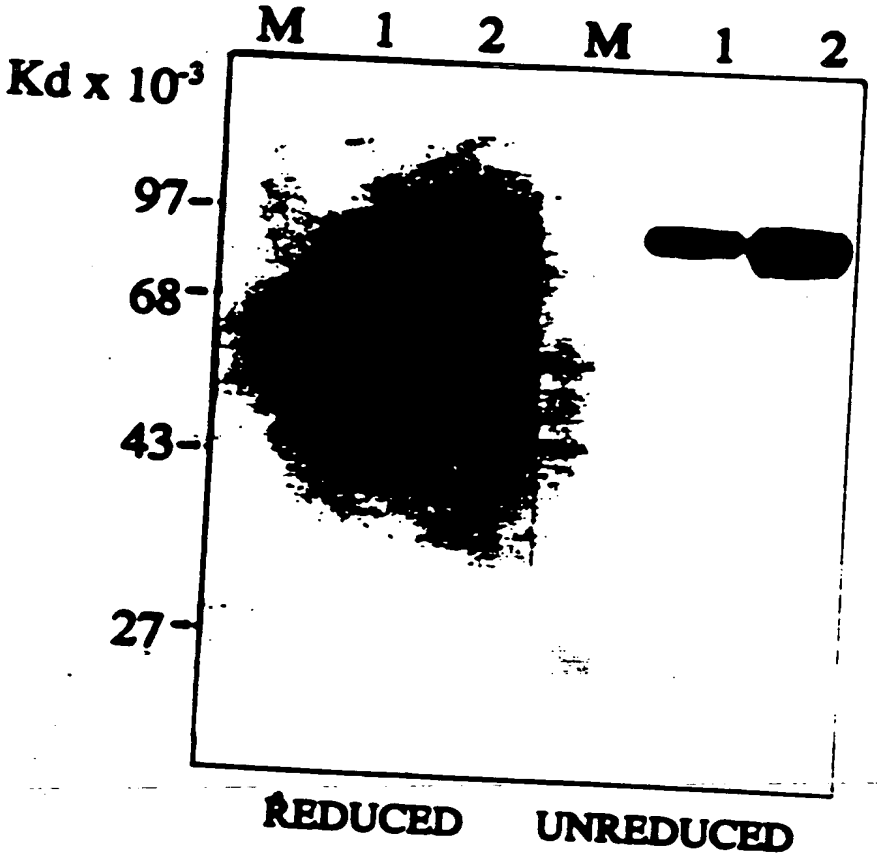


Figure 7

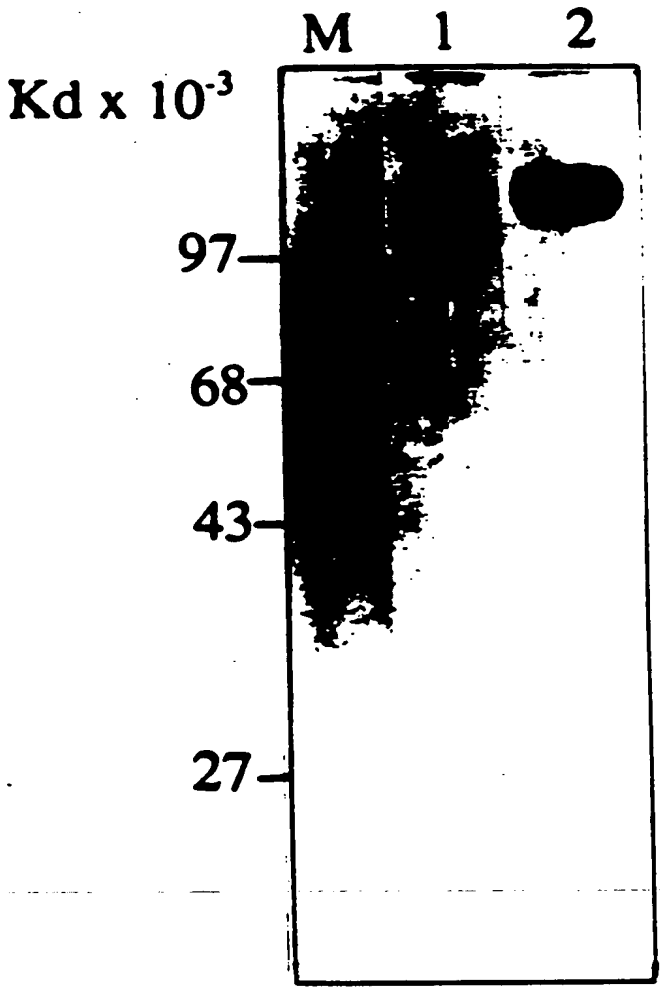
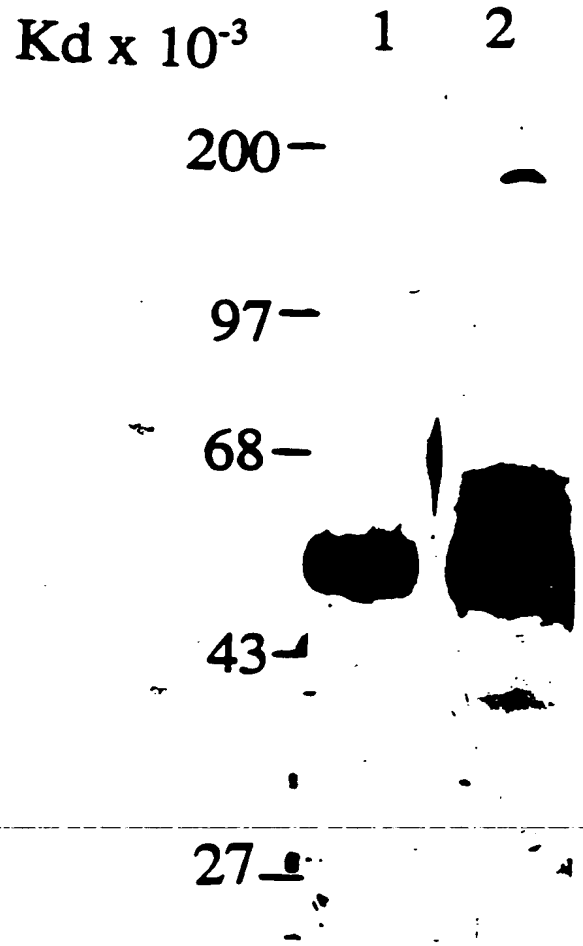


Figure 8



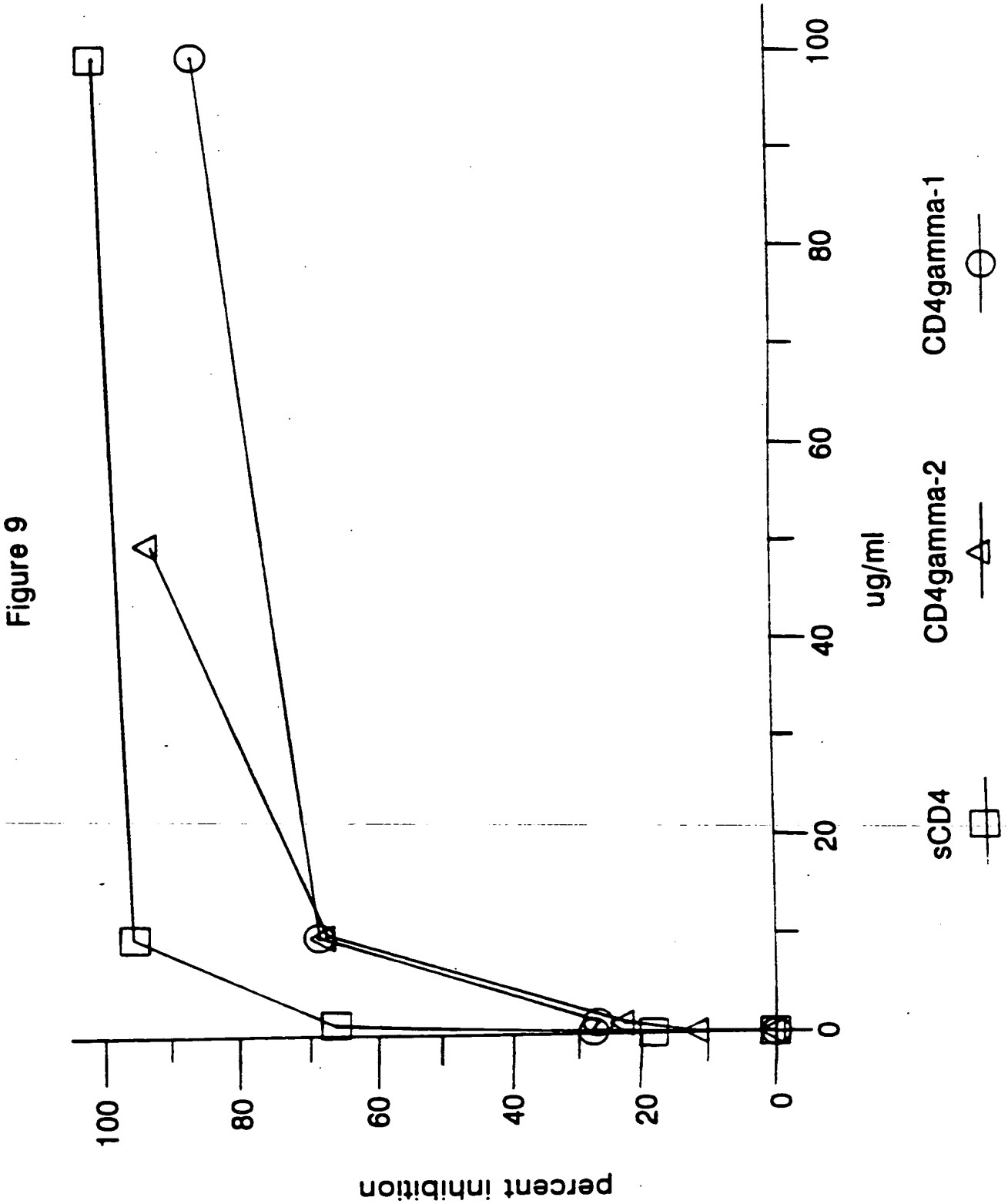


Figure 9
percent inhibition
0
20
40
60
80
100
ug/ml
0
20
40
60
80
100
sCD4
CD4gamma-2
CD4gamma-1

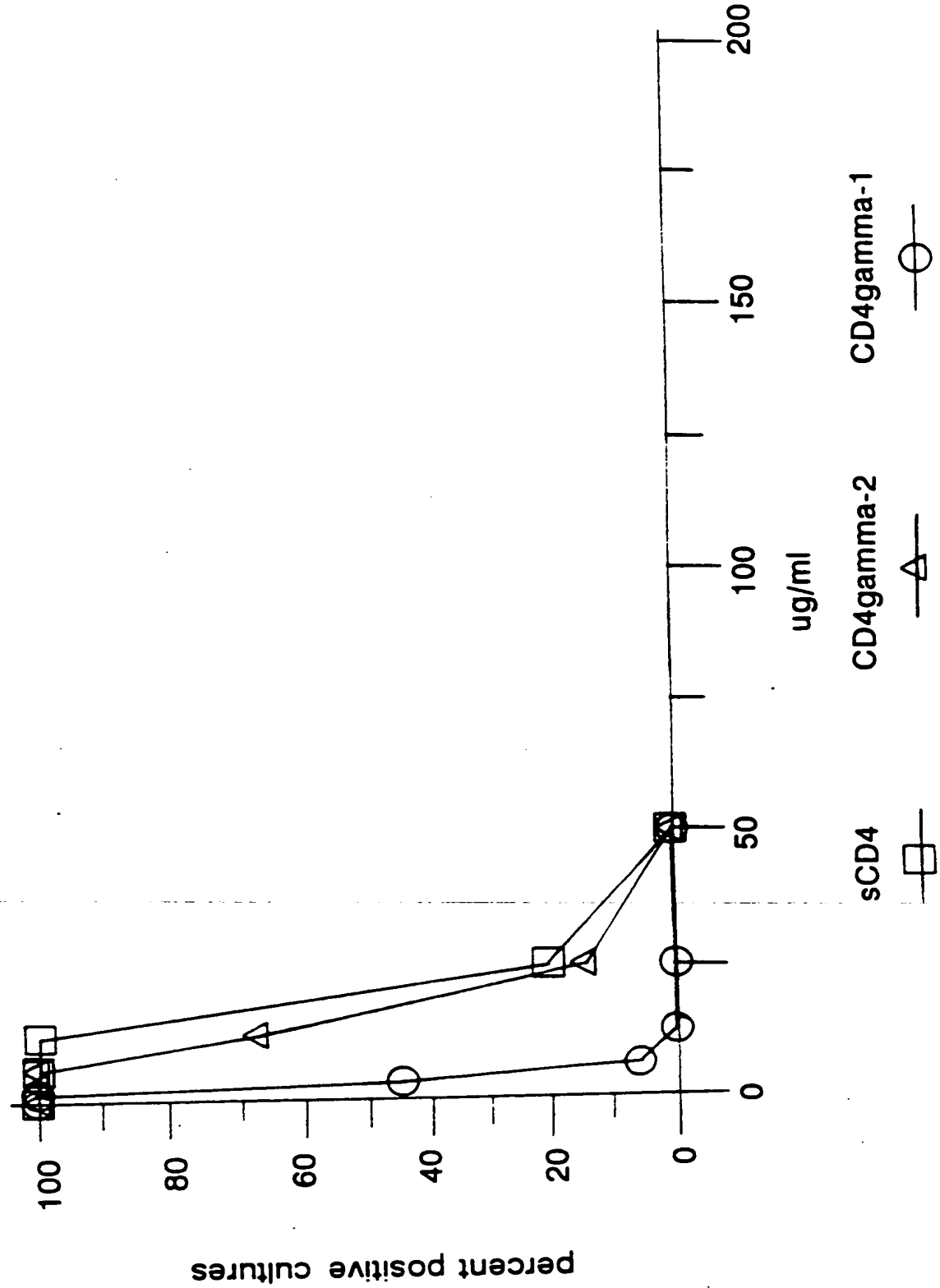

percent positive cultures
100
80
60
40
20
0
0
50
100
150
200
ug/ml
sCD4
CD4gamma-2
CD4gamma-1

Figure 10

Figure 11

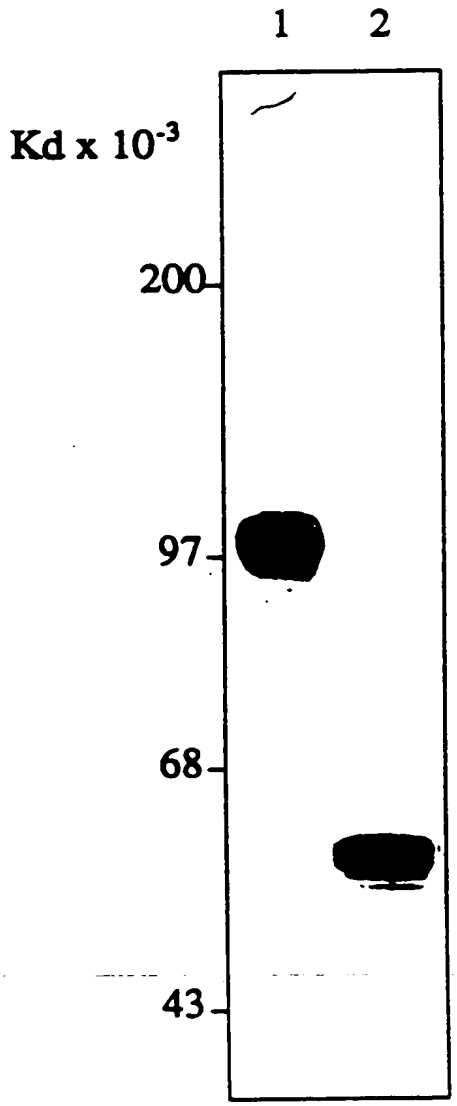


Figure 12B

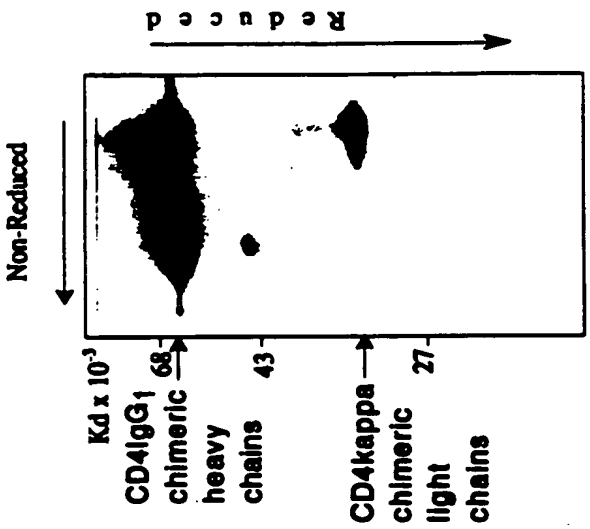


Figure 12A